

Please replace the paragraph at page 9, lines 9-23 with the following:

NS4 cells were negative when stained for neuronal markers, such as ~~-, beta-III-tubulin~~ beta-III tubulin or NeuN. Antibodies recognizing ~~-, beta-tubulin~~ beta-tubulin isotype III (beta-III-tubulin) are commercially available (*for example*, mouse monoclonal antibodies from Sigma Chemicals, St. Louis MO). Antibody to Neuro-Specific Nuclear Protein (NeuN) reacts with most neuronal cell types throughout the nervous system, is available from Chemicon (Temecula CA). The antibody is neuron-specific; no staining of glia is observed. Other neuronal markers include the homeobox-related murine gene ~~Meis2~~ MEIS2 labels the lateral somitic compartment and derivatives during early mouse embryogenesis and later becomes a marker for the dorso-ectodermal region, overlying cells of the paraxial mesoderm. ~~Meis2~~ MEIS2 is also highly expressed in specific areas of the developing central nervous system from embryonic day 9 (E9) onward. In later developmental stages, a strong expression is detectable in differentiating nuclei and regions of the forebrain, midbrain, hindbrain, and spinal cord. (*see*, Toresson *et al.*, 126 (6) Development 1317-1326 (1999)). Another neuronal marker is the DLX homeobox gene, which is expressed in distinct regions of the embryonic forebrain, including the striatum, neocortex and retina (*see*, Eisenstat *et al.*, 414(2) J. Comp. Neurol. 217-37 (1999))

Please replace the paragraph at page 13, line 24 through page 14, line 6 with the following:

NS4 cells can be differentiated into neurons by culturing the NS4 cells on a fixed substrate in a culture medium that is free of the proliferation-inducing growth factor and serum. After removal of the proliferation-inducing growth factor and the serum, the NS4 cells begin to differentiate into neurons. At this stage the culture medium may contain serum such as 0.5-1.0% fetal bovine serum (FBS). However, if defined conditions are required, serum is not used. Within 2-3 days, many of the NS4 cell progeny begin to lose immunoreactivity for GFAP and nestin and begin to express antigens specific for neurons (*e.g.*, ~~-, β-tubulin~~ β-tubulin III). Under the same conditions, NS4 cells can be differentiated into mature astrocytes by culturing the cells on a fixed substrate in a culture medium that is free or deficient of serum. After removal of the serum, the cells flatten, and begin to differentiate into glia. Cells exhibit the astroglial morphology and lose

immunoreactivity for nestin and begin to express GFAP in a fibrillary pattern characteristic for astrocytes.

Please replace the paragraph at page 14, lines 7-16 with the following:

Differentiation of the NS4 cells can also be induced by any method known in the art which activates the cascade of biological events which lead to growth, which include the liberation of inositol triphosphate and intracellular  $\text{Ca}^{2+}$ , liberation of diacyl glycerol and the activation of protein kinase C and other cellular kinases, and the like. Treatment with phorbol esters, differentiation-inducing growth factors and other chemical signals can induce differentiation. Instead of proliferation-inducing growth factors for the proliferation of NS4 cells (*see above*), differentiation-inducing growth factors can be added to the culture medium to influence differentiation of the NS4 cells. Differentiation inducing growth factors include NGF, platelet-derived growth factor (PDGF), thyrotropin releasing hormone (TRH), transforming growth factor betas (~~TGF~~, sTGF), insulin-like growth factor (IGF-1) and the like.

Please replace the paragraph at page 14, line 23 through page 15, line 16 with the following:

Immunocytochemistry can also be used to identify neurons, by detecting the expression of neurotransmitters or the expression of enzymes responsible for neurotransmitter synthesis. For the identification of neurons, antibodies can be used that detect the presence of acetylcholine (ACh), dopamine, epinephrine, norepinephrine, histamine, serotonin or 5-hydroxytryptamine (5-HT), neuropeptides such as substance P, adrenocorticotrophic hormone, vasopressin or anti-diuretic hormone, oxytocin, somatostatin, angiotensin II, neurotensin, and bombesin, hypothalamic releasing hormones such as TRH and luteinizing releasing hormone, gastrointestinal peptides such as vasoactive intestinal peptide (VIP) and cholecystokinin (CCK) and CCK-like peptide, opioid peptides such as endorphins and enkephalins, prostaglandins, amino acids such as GABA, glycine, glutamate, cysteine, taurine and aspartate, and dipeptides such as carnosine. Antibodies to neurotransmitter-synthesizing enzymes can also be used such as glutamic acid decarboxylase (GAD) which is involved in the synthesis of GABA, choline acetyltransferase (ChAT) for ACh synthesis, dopa decarboxylase (DDC) for dopamine,

~~dopamine, hydroxylase~~ dopamine- $\beta$ -hydroxylase (DBH) for norepinephrine, and amino acid decarboxylase for 5-HT. Antibodies to enzymes that are involved in the deactivation of neurotransmitters may also be useful such as acetyl cholinesterase (AChE) which deactivates ACh. Antibodies to enzymes involved in the reuptake of neurotransmitters into neuronal terminals such as monoamine oxidase and catechol-o-methyl transferase for dopamine, for 5-HT, and GABA transferase for GABA may also identify neurons. Other markers for neurons include antibodies to neurotransmitter receptors such as the AChE nicotinic and muscarinic receptors, adrenergic receptors, the dopamine receptor, and the like. Cells that contain a high level of melanin, such as those found in the substantia nigra, could be identified using an antibody to melanin.

Please replace the paragraph at page 18, lines 12-24 with the following:

Survival of the NS4 cell graft in the living host can be examined using various non-invasive scans such as computerized axial tomography (CAT scan or CT scan), nuclear magnetic resonance or magnetic resonance imaging (NMR or MRI), or positron emission tomography (PET) scans. Post-mortem examination of graft survival can be done by removing the neural tissue, and examining the affected region macroscopically and microscopically. Cells can be stained with any stains visible under light or electron microscopic conditions, more particularly with stains that are specific for neurons and glia. Particularly useful are monoclonal antibodies that identify neuronal cell surface markers such as the M6 antibody that identifies mouse neurons. Also useful are antibodies that identify neurotransmitters (such as GABA, TH, ChAT, and substance P) and to enzymes involved in the synthesis of neurotransmitters (such as GAD). Transplanted cells can also be identified by prior incorporation of tracer dyes such as rhodamine-labeled or fluorescein-labeled microspheres, fast blue, bisbenzamide, or retrovirally introduced histochemical markers such as the *lacZ* gene, which produces,  $\alpha$ -galactosidase.

Please replace the paragraph at page 20, line 22 through page 21, line 8 with the following:

NS4 cells can also be genetically modified using calcium phosphate transfection techniques. For standard calcium phosphate transfection, the cells are mechanically dissociated

into a single cell suspension and plated on tissue culture-treated dishes at 50% confluence (50,000-75,000 cells/cm<sup>2</sup>) and allowed to attach overnight. In one protocol, the modified calcium phosphate transfection procedure is performed as follows: DNA (15-25 µg) in sterile TE buffer (10 mM Tris, 0.25 mM EDTA, pH 7.5) diluted to 440 µL with TE, and 60 µL of 2 M CaCl<sub>2</sub> (pH to 5.8 with 1M HEPES buffer) is added to the DNA/TE buffer. A total of 500 µL of 2x ~~HeBS~~ HBS (HEPES-Buffered saline; 275 mM NaCl, 10 mM KCl, 1.4 mM Na<sub>2</sub> HPO<sub>4</sub>, 12 mM dextrose, 40 mM HEPES buffer powder, pH 6.92) is added dropwise to this mix. The mixture is allowed to stand at room temperature for 20 min. The cells are washed briefly with 1x ~~HeBS~~ HBS and 1 ml of the calcium phosphate precipitated DNA solution is added to each plate, and the cells are incubated at 37°C for 20 min. Following this incubation, 10 ml of “NS4 Complete Medium” is added to the cells, and the plates are placed in an incubator (37°C, 9.5% CO<sub>2</sub>) for an additional 3-6 hours. The DNA and the medium are removed by aspiration at the end of the incubation period, and the cells are washed 3 times with “NS4 Complete Growth Medium” and then returned to the incubator.

Please replace the paragraph at page 21, lines 9-16 with the following:

When the genetic modification is for the production of a biologically active substance, the substance can be one that is useful for the treatment of a given CNS disorder. NS4 cells can be genetically modified to express a biologically active agent, such as growth factors, growth factor receptors, neurotransmitters, neurotransmitter synthesizing genes, neuropeptides, and chromaffin granule amine transporter. For example, it may be desired to genetically modify cells so they secrete a proliferation-inducing growth factor or a differentiation-inducing growth factor. Growth factor products useful in the treatment of CNS disorders include NGF, BDNF, the neurotrophins, CNTF, amphiregulin, FGF-1, FGF-2, EGF, TGFα, ~~TGF<sub>β</sub>~~, TGF, PDGF, IGFs, and the interleukins.

Please replace the paragraph at page 25, lines 1-9 with the following:

Other regulatory factors include heparan sulfate, ~~TGF<sub>β</sub>~~, TGF, activin, BMP-2, CNTF, retinoic acid, ~~TNF~~, ~~MIP-1~~, ~~MIP-1~~, ~~MIP-2~~, acid, TNF, MIP-1, MIP-2, NGF, PDGF,

interleukins, and the Bcl-2 gene product. Other factors having a regulatory effect on stem cell proliferation include those that interfere with the activation of the *c-fos* pathway (an intermediate early gene, known to be activated by EGF), including phorbol 12 myristate 13-acetate (PMA; Sigma), which up-regulates the *c-fos* pathway and staurosporine (Research Biochemical International) and CGP-41251 (Ciba-Geigy), which down regulate *c-fos* expression and factors, such as tyrphostin (Fallon *et al.*, 11(5) Mol. Cell Biol. 2697-2703 (1991)) and the like, which suppress tyrosine kinase activation induced by the binding of EGF to its receptor.

Please replace the paragraph at page 25, line 21 through page 26, line 6 with the following:

Using these screening methods, one of skill in the art can screen for potential drug side-effects on pre-natal and post-natal CNS cells by testing for the effects of the biological agents on neural cell proliferation and differentiation or the survival and function of differentiated CNS cells. The proliferated NS4 cells are typically plated at a density of about  $5-10 \times 10^6$  cells/ml. If it is desired to test the effect of the biological agent on a particular differentiated cell type or a given make-up of cells, the ratio of neurons to glial cells obtained after differentiation can be manipulated by separating the different types of cells. Astrocytes can be panned out after a binding procedure using the RAN 2 antibody (available from ATCC). Tetanus toxin (available from Boehringer Ingelheim) can be used to select out neurons. By varying the trophic factors added to the culture medium used during differentiation it is possible to intentionally alter the phenotype ratios. Such trophic factors include EGF, FGF, BDNF, CNTF, ~~TGF~~, TGF, GDNF, and the like. For example, FGF increases the ratio of neurons, and CNTF increases the ratio of oligodendrocytes. Growing the cultures on beds of glial cells obtained from different CNS regions can also affect the course of differentiation.

Please replace the paragraph at page 28, lines 19-29 with the following:

After a couple of passages, the parental culture shows >95% GFAP and nestin immunoreactivity. In addition, the cultures express the radial glial marker RC2. After switching to serum-free medium and removing the EGF (differentiating condition), the cells change morphology and staining pattern to become as much as 36% neurons (as determined by

morphology and  $\beta$ -tubulin III). This finding could be reproduced with cells at least 15 passages old. The fact that the neurons had arisen from GFAP<sup>+</sup> parental cells was established beyond doubt by using the transgenic mouse cultures. Many of the  $\beta$ -tubulin III immunoreactive cells also expressed EGFP indicating that they had been GFAP<sup>+</sup> at an earlier time point. Last, some cells retained their molecular identity and express transcription factors typical of differentiating neurons in the lateral ganglionic eminence (*e.g.* DLX and Meis2 MEIS2), showing that the cells are specified progenitors.

Please replace the paragraph at page 29, lines 15-19 with the following:

After several passages (>4), parental cells were investigated for their morphology and expression of GFAP, nestin, and  $\beta$ -tubulin III. Similar to the mouse cultures (EXAMPLE 1), human cells were placed in serum-free medium without growth factors and the differentiation studied with morphology and immunocytochemistry. Adherent human cell cultures could be established in a similar manner to mouse cultures (EXAMPLE 1).

Please replace the paragraph at page 30, line 26 through page 31, line 8 with the following:

In this EXAMPLE, cultures of dissociated cells prepared from lateral ganglionic eminence of the mouse embryonic day 15-17 (E15-17) forebrain were established in a medium including epidermal growth factor (EGF) and serum, to obtain propagating attached cultures with a high content of astroglia-like cells. This EXAMPLE is to determine the long-term characteristics of cells cultured under these conditions. The cultures were passaged at confluency, and growth rate, morphology and phenotypic properties (*e.g.* GFAP immunoreactivity) were assessed after the subsequent passages. The cultured cells had the morphology of astroglial cells, with the vast majority of the cells immunoreactive for GFAP (around 90%), as well as for the intermediate filament marker nestin. The cells were also positive for the mouse-specific neural antibodies M2 and M6. The cells were negative when stained for the neuronal marker  $\beta$ -tubulin III.

Please replace the paragraph at page 31, lines 9-26 with the following:



*Dissociation.* Lateral ganglionic eminence tissue was retrieved mainly from E15, but in a few cases also from E16-17, mouse embryos of timed pregnant mice. With the embryos immediately placed in a 1:1 mixture of Dulbecco's minimum essential medium (DMEM) and F12 (Gibco), the brains were removed, the cortex unfolded after a medial parasagittal cut and the underlying lateral ganglionic eminence dissected out bilaterally, using the method of Olsson *et al.*, 69 Neuroscience 1169-82 (1995). The tissue pieces, collected from one litter of embryos at a time, were then placed in a 0.1% trypsin (Worthington Biochemical Corporation)/0.05% DNase (Sigma) solution in DMEM/F12 and incubated for 20 min at 37°C. Following rinses in DMEM/F12 with 0.05% DNase, the pieces were mechanically dissociated by repeated gentle trituration through the tips of two Eppendorf pipettes with decreasing diameters and centrifuged for 5 min at 600 rpm. The pellet was then resuspended and plated onto uncoated T75 flasks (Falcon), with a medium containing DMEM/F12 supplemented with 10% Fetal Bovine Serum (FBS; Sigma), EGF (20 ng/ml, human recombinant; R & D Systems), a defined hormone and salt mixture including 20 µg/ml insulin, 100 µg/ml transferrin, 20 nM progesterone, 60 µM putrescine and 30 nM sodium selenite (all from Sigma Chemicals, St. Louis CO; *see*, Weiss *et al.*, 16 J. Neurosci. 7599-609 (1996)) and 1% AAS (antibiotic antimycotic solution; Sigma). The cultures were maintained at 37°C with 95% air and 5%  $\text{CO}_2$ , with the medium changed every 2-3 days, and the cells passaged (or frozen down using DMSO and serum) at confluency.

Please replace the paragraph at page 33, line 20 through page 34, line 7 with the following:

*Immunoreactivity.* GFAP-immunocytochemistry, performed after each of the first five passages and after passage 18, revealed that around 75% of the cells were GFAP<sup>+</sup> already after passage two, and with approximately 90% ~~GFAP<sup>+</sup>~~ GFAP<sup>+</sup>-immunoreactive cells after passage five, and also after passage 18 (FIG. 4). A similar proportions of the cells also expressed the intermediate filament nestin, both at the early and late passages. The mouse-specific neural markers M2 and M6 were also detected in the majority of the cells, overlapping with the GFAP and nestin immunoreactivities, but with a reduced expression of M2 at the later passages, and with M6 in general expressed at lower levels than M2. No or only occasionally,  $\beta$ -tubulin III<sup>+</sup> cells were detected at either passage five or 18. For immunocytochemistry, 100,000 cells were

plated in uncoated 4-well plates (NUNC) after each passage, and after attachment fixed in 4% paraformaldehyde (PFA). After rinses with potassium phosphate buffered saline (KPBS), the cultures were preincubated with 5% normal serum raised in the same species as the secondary antibody, in 0.02 M KPBS for 1 hr at room temperature (RT). Following incubation with primary antibodies (overnight at 4°C), the cultures were rinsed three times in 0.02 M KPBS (with 5% serum), and incubated with a biotinylated secondary antibody (2 hrs, 1(T), rinsed in KPBS and incubated with an avidin-biotin-peroxidase complex (Vectastain-Elite ABC Kit PK-6 100) using 3,3-diaminobenzidine as chromogen (25 mg/ml; Sigma).

Please replace the paragraph at page 34, line 21 through page 35, line 13 with the following:

To generate neurons, cells were proliferated as in EXAMPLES 1 or 2 on uncoated tissue culture plastic. Three days after plating, a medium switch was performed from the expansion medium to the same medium minus the serum and EGF. In some cases, a sequential switch was performed, where first serum was removed and then EGF a few days later. Cultures for indirect immunocytochemistry, were kept in the serum-free medium (without EGF) for 4-8 days before fixation in 4% paraformaldehyde in PBS. Following a 10 min fixation, coverslips were washed three times in PBS and immunostained for neuronal and glial markers (*e.g.* beta-tubulin III, GFAP, nestin, and RC2). Coverslips were incubated with primary antiserum in PBS/10% normal goat serum, 0.3% TRITON-X-100 for two hours at 37°C. Coverslips were washed 3x in PBS and incubated with labeled secondary antibodies for 30 min at 37°C. Coverslips were then washed 3x in PBS, rinsed with water and placed on glass slides. Between 17- 36% of the cells derived from cell cultures established from different dissections including human LGE, were neurons as determined by morphology and beta-tubulin III staining. To further examine the neuronal differentiation, ICC staining for GABA and calbindin were done indicating that most of the cells formed a GABA-ergic neuronal phenotype. Furthermore, the cells showed other evidence of retention of the striatal specification such as the expression of the marker DLX1 and MEIS2 but not PAX6 and NKX2.1, markers of cortical and MGE neurons respectively. The neuronal phenotype and function was further confirmed by electrophysiology which demonstrated electrochemical activity characteristic of neurons. During differentiation, many cells co-labeled with both ~~beta-III-tubulin~~ beta-tubulin III



and GFAP. With further differentiation mature neuronal and astrocytic phenotypes and separate ~~beta-II-tubulin~~ beta-tubulin III and GFAP immunoreactivity were observed.

Please replace the paragraph at page 35, lines 24-26 with the following:

The neurons we generate turn on, not only the neuron-specific marker-, beta-tubulin III, but also ~~Meis2~~ MEIS2 and DLX, transcription factors specific for the regions where the glia were dissected from.

Please replace the paragraph at page 35, line 27 through page 36, line 4 with the following:

To determine whether the neurons actually derive from glial cells we used cell cultures from the GFAP-tva mouse. These mice express the receptor for the RCAS-virus, tva, under the GFAP promoter (*see*, EXAMPLE 1). Thus, only GFAP expressing cells can be infected. After infection with an RCAS(A)GFP (green fluorescent protein) virus, GFP cells were found that were also-, beta-tubulin III<sup>+</sup> and had a neuronal morphology. These results show that a subpopulation of GFAP<sup>+</sup> cells in multipassage glial cultures derived from the ventral telencephalon are indeed neuronal precursors.

Please replace the paragraph at page 37, lines 11-20 with the following:

*Method of transplantation into neonates.* Cell suspensions from passages 4-6 (“*Neonate a*”) and passage 18 (“*Neonate b*”) cultures (from EXAMPLE 4) were injected into the striatum bilaterally in 27 neonatal rats and unilaterally into five neonates, with a cell density adjusted to 25 000 cells/p1 or 100,000 cells/0.1pl. The injections were made from glass capillaries attached to a Hamilton syringe and in all cases the neonates were placed in a neonatal frame during surgery (*see*, Cunningham & McKay, 47 J. Neurosci. Methods 105-14 (1993)). In total, 2 l/neonatal striatum were injected at the following coordinates:

(1) A +0.5; L +/-2.2; V (a) -3.0, (b) -2.5;

(2) A +0.9; L +/-1.9; V (a) -3.0, (b) -2.5;

with bregma and lambda at the same horizontal level.